PNAS
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Supplementary Information for
Arabidopsis Group C Raf-like protein kinases negatively regulate abscisic acid signaling and are direct substrates of SnRK2

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Plant Materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used as WT. T-DNA insertional mutant lines, *raf36-1* (GK-459C10), *raf36-2* (SALK_044426C) and *raf22* (SALK_105195C) were obtained from ABRC or GABI-Kat. The *raf36-1raf22* double mutant was generated by crossing *raf36-1* and *raf22*. The *SRK2E/OST1* knockout mutant, *srk2e* (SALK_008068), was used as previously described (1). The *srk2dsrk2e* double mutant was generated by crossing *srk2d* (GABI-Kat 807G04) and *srk2e*, and the *raf36-1srk2dsrk2e* triple mutant was established by crossing *srk2dsrk2e* double mutant and *raf36-1*. Seeds of wild-type, mutants or transgenic plants were sterilized, and sown on germination medium (GM) agar plates as described (2). After vernalization at 4°C in the dark for 4 days, they were incubated in a growth chamber under a continuous light condition of 100 μmol m^{-2} s^{-1} photon flux density (NEC fluorescent lamps FHF32EX-N-HX-S) at 22°C for indicated periods. To test ABA sensitivity, seeds were sown on GM agar medium with or without 0.5 μM ABA (Sigma, MO). Germination and greening rate were scored daily for 14 days according to previous study (3).

Plasmids

In this study, vector constructions were performed with Gateway cloning technology (Thermo Fisher Scientific, MA) unless otherwise noted. SRK2D, SRK2E and SRK2I cDNAs were previously cloned into pENTR/D-TOPO vector (Thermo Fisher Scientific) (2). In addition, Raf36, Raf36 N (amino acid residues 1-206), Raf36 KD+C (207-525), Raf43, Raf22 and Raf28 cDNAs were cloned into pENTR/D-TOPO or pENTR1A vector and sequenced. Site-directed mutagenesis was carried out as previously described (4). Those cDNAs were transferred to destination vectors, such as pGBKT7 and pGADT7 (Takara Bio, Japan), pSITE-nEYFP-C1 and pSITE-cEYFP-N1 (ABRC), pGEX6p-2 (GE healthcare, IL), pBE2113-GFP (1) and pGreen0029-HA (2). FLAG-tagged SRK2I was cloned into pEarleyGate 100 (5) to prepare *35Spro:FLAG-SRK2I* construct. For the *Raf36pro:Raf36-3xFLAG* or *Raf36pro:Raf36 K234N-3xFLAG* construct, the putative *Raf36* promoter (2,729 bp upstream sequence of the *Raf36* coding region) was amplified using specific primers with Gateway attB adaptor sequences and cloned into pDONR P4-P1R vector (Invitrogen) using Gateway BP clonase II (Invitrogen). In addition, the *3xFLAG* sequence was inserted into the pENTR/D-TOPO *Raf36* construct by inverse PCR followed by self-ligation. The resulting vectors, pDONR P4-P1R harboring *Raf36pro* and pENTR/D-TOPO harboring *Raf36-3xFLAG* or *Raf36 K234N-3xFLAG*, were reacted with R4pGWB501 (6) using Gateway LR clonase II (Invitrogen). For the *2x35Spro:Raf36-3xFLAG* construct, pDONR P4-P1R harboring *2x35S* promoter and pENTR/D-TOPO harboring *Raf36-3xFLAG* were cloned into R4pGWB501 (6). The primers used in this section are listed in Supplemental Dataset 5.

Transgenic Plants

We prepared pBE2113 containing GFP-tagged Raf36 or Raf22, pGreen0029 containing hemagglutinin (HA)-tagged Raf36, R4pGWB501 containing 3xFLAG-tagged Raf36 and pEarleyGate 100 containing FLAG-tagged SRK2I. Each plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 (for pBE2113, R4pGWB501 and pEarleyGate 100) or GV3101 (pSOUP) (for pGreen0029) by electroporation, and then transformed into Arabidopsis plants as described (7). Transgenic plants were selected on GM agar medium containing 50 μg/ mL kanamycin, 25 μg/ mL hygromycin or 10 μg/ mL Basta with 200 μg/ mL claforan. Expression of transgenes was confirmed by RT-PCR or western blotting.

AlphaScreen®

The AlphaScreen® (Amplified Luminescent Proximity Homogeneous Assay) was carried out using an AlphaScreen® FLAG® (M2) Detection Kit (Perkin Elmer, MA), which can detect protein-protein interactions with no washing steps during the assay. For screening of SnRK2-interacting MAPKKKs, 15 MAPKKKs were selected at random from the entire family members. Then, the C-terminal FLAG (DYKDDDDK)-tagged MAPKKK proteins were expressed in wheat germ extract (WGE) from *in vitro* synthesized mRNA obtained from PCR-amplified cDNAs (8). The N-terminal biotinylated SnRK2 proteins, such as SRK2D, E and I, were synthesized in WGE supplemented with 45 μM d-biotin (Cosmo Bio, Japan) and Biotin Ligase A (BirA) (Cosmo Bio, Japan), and subsequently subjected to ultrafiltration using Amicon® Ultra-0.5 30K centrifugal filter devices

(Millipore, MA) for the removal of the remaining d-biotin. The protein quality (i.e., efficient synthesis with the expected molecular weight) of FLAG-tagged MAPKKKs and biotinylated SnRK2s was confirmed by western blotting with an anti-FLAG antibody (WAKO, cat. no. 014-22383, lot no. SAR0168) and streptavidin-HRP (Cell Signaling technology, cat. no. 3999), respectively. Then, FLAG-tagged MAPKKK-synthesized mixture and biotinylated SnRK2-synthesized mixture was diluted 5.6-fold and 10-fold with sterilized water, respectively. To detect the interactions between FLAG-tagged MAPKKKs and biotinylated SnRK2s, 2 µL of each protein-synthesized mixture was mixed and reacted in a 25 µL reaction buffer containing 0.1 µL of anti-FLAG conjugated AlphaScreen Acceptor beads, 0.1 µL of streptavidin-coated Donor beads, 0.01% (w/v) Tween-20 and 0.1% (w/v) bovine serum albumin (BSA) in sterilized water-diluted control buffer provided in the kit and then incubated in a 384-well microplate at 21°C for 12 h. The AlphaScreen® luminescence was detected with the infinite® M1000 Pro (TECAN, Switzerland). WGE with no expressed proteins was employed as negative control (NC) to estimate the luminescence caused by endogenous wheat germ proteins.

Yeast two-hybrid analysis

Yeast two-hybrid analysis was employed using the MatchMaker GAL4 Two-Hybrid System 3 (Takara Bio, Japan) as previously described (2). *Saccharomyces cerevisiae* strain AH109 was co-transformed with various pairs of pGBKT7 vectors harboring SnRK2s (i.e., SRK2D, SRK2E and SRK2I) and pGADT7 vectors harboring Raf kinases (i.e., Raf36, Raf43, Raf22 and Raf28). A single colony for each transformant grown on SD/-leucine (L)/-tryptophan (W) media was incubated in liquid media, and then evaluated on SD media supplemented with or without 3-amino-1,2,4-triazole (3-AT) and lacking combinations of amino acids leucine (L), tryptophan (W) and histidine (H), as follows: -LW, -LWH, -LWH +10 mM 3-AT, -LWH +50 mM 3-AT or on SD media lacking L, W, H, and adenine (A), as follows: -LW, -LWH, -LWHA. The plates were incubated at 30°C for the optimal period. For X-Gal overlay assay, colonies were immersed by chloroform for 5 min. After eliminating chloroform, plates were overlaid with 1% melting agarose, 1mg/mL X-β-Gal and 100 mM KPO_4 (pH7.0), and further incubated at 30°C for 24 h.

Microscopy analyses of fluorescent proteins

To perform *Agrobacterium*-mediated bimolecular fluorescence complementation (BiFC) assay, pSITE-nEYFP-C1 vectors harboring SnRK2s (i.e., SRK2D, SRK2E and SRK2I) or pSITE-cEYFP-N1 vectors harboring Raf-like kinases (i.e., Raf36, Raf43, Raf22 and Raf28) were introduced to *A. tumefaciens* strain GV3101(p19) by electroporation. A single colony for each transformant was cultured in LB media, and the media was substituted by 1/2 GM liquid media supplemented with 0.1 mM acetosyringone. SnRK2 and Raf transformants were mixed with various pairs, and then infiltrated into *Nicotiana benthamiana* leaves. Complemented YFP fluorescence of each samples was observed in epidermall cells of *N. benthamiana* at 3 days after infiltration with a fluorescence microscope BX53 (Olympus, Japan). For analysis of subcellular localization of Raf36-GFP, mesophyll cells of 2-week-old transgenic Arabidopsis plants expressing Raf36-GFP were observed with a confocal microscope SP8X (Leica Microsystems) with the time-gating method (9), which completely eliminates chlorophyll autofluorescence when GFP imaging. GFP fluorescence was observed with 484 nm excitation and 494-545 nm emission with a gating time of 0.3–12.0 nsec. Chlorophyll autofluorescence was separately observed with 554 nm excitation and 640–729 nm emission.

Co-immunoprecipitation (Co-IP) assay

The Co-IP assay was performed using 3-week-old *35S:Raf36-HA* and *35S:Raf36-HA/ 35S:FLAG-SRK2I* plants (approximately 30 seedlings for each) grown on GM agar plates and treated or not treated with 50 µM ABA for 30 min. The seedlings were ground to a powder in liquid nitrogen, and crude proteins were extracted in 4 mL of extraction buffer [20 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 0.5% TritonX-100, 1 mM Na_3VO_4, 25 mM NaF and 1% Protease Inhibitor Cocktail (Thermo Fisher Scientific)], and subsequently subjected to two-step centrifugation at 17,400 × *g* for 10 min at 4°C to remove the cellular debris. The supernatant was incubated with 4 µL of anti-FLAG (DYKDDDDK) tag antibody (WAKO, cat. no. 014-22383, lot no. SAR0168) for 4 h at 4°C followed by a 1-h incubation with 100 µL of 50% slurry of Protein G

Sepharose 4 Fast Flow (Cytiva) at 4°C. Immunocomplexes were washed three times with 1 mL of the extraction buffer without the protease inhibitor cocktail. After washing, the proteins were eluted with 50 µL of 100 mM glycine-HCl (pH 3.0) at room temperature and neutralized with 6 µL of 1 M Tris-HCl (pH 9.0). Then, aliquots were separated by 10% SDS-PAGE gel and analyzed by western-blot analysis using anti-HA [16B12] antibody (BioLegend, cat. no. 901513, lot no. B274467) or anti-FLAG (DYKDDDDK) tag antibody (WAKO, cat. no. 014-22383, lot no. SAR0168).

Preparation of Recombinant Proteins

DNA fragments of Raf36, Raf36 N (1-206 aa), Raf36 KD+C (207-525), Raf36 KD (207-467), Raf36 N (1-156), Raf36 N (1-140), Raf22, Raf43, Raf28, SRK2D, SRK2E and SRK2I were amplified from cDNAs, and they were fused in-frame to the EcoRV site of pMAL-c5X vector (New England Biolabs, MA) by using In-Fusion HD Cloning Kit (TaKaRa, Japan). Amino acid substitutions, such as Raf36 K234N, Raf36 S145A, Raf36 S145D, Raf36 S145A S157A, Raf36 S145D S157D, Raf22 K157N, Raf22 S81A K157N, Raf43 K228N, Raf28 K158N and SRK2E K50N, were introduced by site-directed mutagenesis. SRK2E and SRK2E K50N cDNAs were cloned into pGEX6p-2 vector (GE healthcare, IL) by Gateway LR clonase II. In addition, to identify phosphorylation sites in Raf36, six types of 30-amino-acid peptides with mutations were designed as Raf36 peptides (134-163) #1-#6 as shown in Fig. 3*C*. Similarly, to confirm Raf22-dependent phosphorylation, nine phosphopeptides and OLE1, a positive control of Raf22 substrates, were designed as 31-amino-acid peptides including the putative phosphorylation site(s). These DNA fragments were fused in-frame to the EcoRI and NotI sites of pGEX4T-3 vector (GE healthcare, IL). The *E. coli* BL21 (DE3) cells carrying expression plasmids (i.e., pMAL-c5X, pGEX 6p-2 or pGEX4T-3) were grown at 37°C, and protein expression was induced by the addition of IPTG (isopropyl β-D-thiogalactopyranoside) to 1 mM at OD_{600} 0.5–0.6 in 2xYT media [1.6% Bacto Tryptone, 1% Yeast Extract and 0.5% NaCl] supplemented with 100 µg/mL ampicillin. After a 10 h incubation at 15°C, the cells were harvested by 4,000 × *g* centrifugation for 6 min at 4°C and the pellet was resuspended in the ice-cold lysis buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% TritonX-100 and 2.5 mg/mL Lysozyme]. Cells were ultra-sonicated on ice four times for 20 s with regular resting intervals. After ultra-sonication, 1,4-dithiothreitol (DTT) was added to 18 mM and the cells were subsequently rotated for 30 min at 4°C. A supernatant was obtained by centrifugation at 17,400 × *g* for 15 min at 4°C. For the affinity purification of the MBP-tagged recombinant proteins, the supernatant was co-incubated with Amylose Resin (New England Biolabs) for 30 min at 4°C. After being washed four times in the washing buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM DTT], the resin was resuspended with an ice-cold MBP-elution buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT and 10 mM maltose] for 2 min. For the affinity purification of the GST-tagged recombinant proteins, the supernatant was co-incubated with Glutathione Sepharose 4B resin (GE healthcare, IL) for 30 min at 4°C. After being washed four times in the washing buffer, the resin was resuspended with an ice-cold GST-elution buffer [50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione] for 2 min. Both MBP-tagged and GST-tagged recombinant proteins were further purified with a Nanosep® 30-kDa size-exclusion column (PALL, NY) for concentration and removal of low-molecular-weight materials. GST-tagged recombinant peptides were also purified with a Nanosep® 10-kDa size-exclusion column (PALL, NY). Whether the protein/peptide of interest was purified was determined by comparison with the predicted molecular weight listed in *SI Appendix, Table S1*. The primers used in this section were listed in Supplemental Dataset 5.

***In vitro* phosphorylation assay**

In vitro phosphorylation assays were performed as described previously with some modifications (2). The recombinant proteins of SnRK2, Raf or substrates were mixed with indicated pair(s) and incubated in 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ or 5 mM $MnCl_2$, 50 µM ATP and 0.037 MBq of [γ-^{32}P] ATP (PerkinElmer, MA) at 30°C for 30 min. Samples were subsequently separated by SDS-PAGE, and phosphorylation levels were detected by autoradiography with BAS-5000 (Fujifilm, Japan).

Identification of Raf36 phosphorylation sites by LC-MS/MS

E. coli recombinant MBP-Raf36 and MBP-SRK2E proteins were prepared as described above. 2 µg of MBP-Raf36 protein was incubated with or without 2 µg of MBP-SRK2E protein in 50 mM Tris-

HCl (pH 7.5), 5 mM $MgCl_2$ and 50 µM ATP at 30°C for 30 min. After trypsin digestion, desalted peptides were analyzed with TripleTOF 5600 LC-MS/MS system (AB-SCIEX). For *in vivo* Raf36 phosphorylation, Arabidopsis 2-week-old transgenic seedlings expressing Raf36-3xFLAG under the control of *Raf36* promoter (*raf36-1* background) were prepared as described above. The seedlings were treated with or without 50 µM ABA for 30 min. Crude extracts were obtained by homogenizing 1.8 g of seedlings in 1.8 mL extraction buffer [20 mM HEPES-KOH (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 25 mM NaF, 1 mM Na_3VO_4, 0.5% TritonX-100, 1% Protease Inhibitor Cocktail (Thermo Fisher Scientific) and 50 µM MG132], and subsequently subjected to two-step centrifugation at 17,400 × *g* for 10 min at 4°C to remove the cellular debris. The supernatant was incubated with 50 µL of anti-DYKDDDDK tag antibody beads (WAKO, cat. no. 018-22783) for 3 h at 4°C. Immunocomplexes were washed three times with 1 mL of the extraction buffer without the protease inhibitor cocktail and MG132. After washing, the beads were resuspended in the 25 µL of the resuspension buffer [100 mM Tris-HCl (pH 9.0), 12 mM sodium deoxycholate, 12 mM sodium *N*-lauroylsarcosinate and 50 mM ammonium bicarbonate]. Then, 100 µL of the digestion buffer [50 mM ammonium bicarbonate and 10 µg/mL trypsin (Promega)] was added and immunocomplexes were digested on beads according to the previous study (10). After tryptic digestion, surfactants were removed by adding ethyl acetate followed by acidification using trifluoroacetic acid (TFA) (11). The peptides were desalted and analyzed with TripleTOF 5600 LC-MS/MS system (AB-SCIEX). The phosphopeptides were identified and quantified based on the extracted ion chromatogram (XIC) peak area using Skyline version 20.2 (MacCoss lab software).

Water loss analysis

To measure leaf water loss, 2-week-old seedlings were transferred from GM agar medium to soil, and the plants were grown under a 16 h/8 h (light/dark) photoperiod of 90 µmol m^{-2} s^{-1} photon flux density (LED white lamps) at 22°C for another 2 weeks. The fully expanded rosette leaves were detached from 4- to 5-week-old plants and placed on weighing dishes. These dishes were kept under the same conditions used for seedling growth on soil, and then their fresh weights were monitored at the indicated times with three replicates per time-point. One replicate consists of 5 individual leaves. Water loss was calculated as a percentage of relative weight at the indicated times versus initial fresh weight.

RNA extraction and qRT-PCR

For quantitative reverse transcription PCR (qRT–PCR) analysis, total RNA was extracted by LiCl precipitation from 1-week-old seedlings treated with 50 µM ABA for indicated periods. 1 µg of total RNA treated with RNase-free DNase I (Nippon Gene, Japan) was used for reverse transcription with ReverTra Ace® reverse transcriptase (TOYOBO, Japan). qRT-PCR analysis was performed using GoTaq® qPCR Master Mix (Promega, WI) with Light Cycler 96 (Roche Life Science, CA). For normalization, *GAPDH* was used as an internal control. The gene-specific primers used for qRT-PCR analysis were shown in Supplemental Dataset 5.

Phosphoproteomic analysis

Following imbibition with 50 µM ABA, 2-week-old Arabidopsis seedling of wild-type (Col-0) and *raf22raf36-1* were used for phosphoproteomic analysis with three biological replicates. Total crude protein was extracted from grounded sample. The phosphoproteomic analyses were performed according to the previous study (4, 12) with minor modifications and with 400 µg of total crude protein. Enriched phosphopeptides by using hydroxy acid-modified metal oxide chromatography (HAMMOC) method (13) were analyzed with a LC-MS/MS system, TripleTOF 5600 LC-MS/MS system (AB-SCIEX). Peptides and proteins were identified using Mascot (Matrix Science, version 2.4.1) against TAIR 10 database (https://www.arabidopsis.org/). Search settings included a precursor mass tolerance of ± 50 parts per million (ppm), a fragment ion mass tolerance of ± 0.05 Da, and strict trypsin specificity, allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionines and phosphorylation of serine, threonine, and tyrosine were set as variable modifications. The Mascot search results were calculated with a score above 33 to be significant (a nonrandom match at $P < 0.05$). A site localization probability of 0.75 was used as the cut-off for localization of phosphorylation site(s).

Skyline version 4.2 (MacCoss lab software) was used for quantification of phosphopeptides based on the extracted ion chromatogram (XIC) peak area. The XIC peak areas were normalized with Levenberg-Marquardt optimization (14). All raw data files were deposited in the Japan Proteome Standard Repository Database (jPOST; JPST000961, https://repository.jpostdb.org/preview/8953133565f5aeb926c47c, access key; 8236). Each phosphoproteomic sample was compared by principal component analysis by using all identified phosphopeptides and their phosphorylation level. For gene ontology (GO) analysis, annotated data was loaded to DAVID (https://david.ncifcrf.gov), and background database was set as TAIR10. Outputted GO terms from DAVID program were visualized using REVIGO (15). Settings used for REVIGO were: medium (0.7) similarity, Uniprot Arabidopsis database and SimRel semantic measure. For motif analysis, 13 amino acids around phosphorylated serine/ threonine/ tyrosine residues were extracted from identified phosphopeptide sequences, and analyzed using the Motif-X algorithm (16), implemented in the rmotifx R package (17). The enriched phosphorylation motifs from rmotifx program were visualized as sequence logos using the texshade package (18). All scripts used in the phosphoproteomics data analyses were uploaded on GitHub (https://github.com/Ume-lab/raf22raf36_phosphoproteome).

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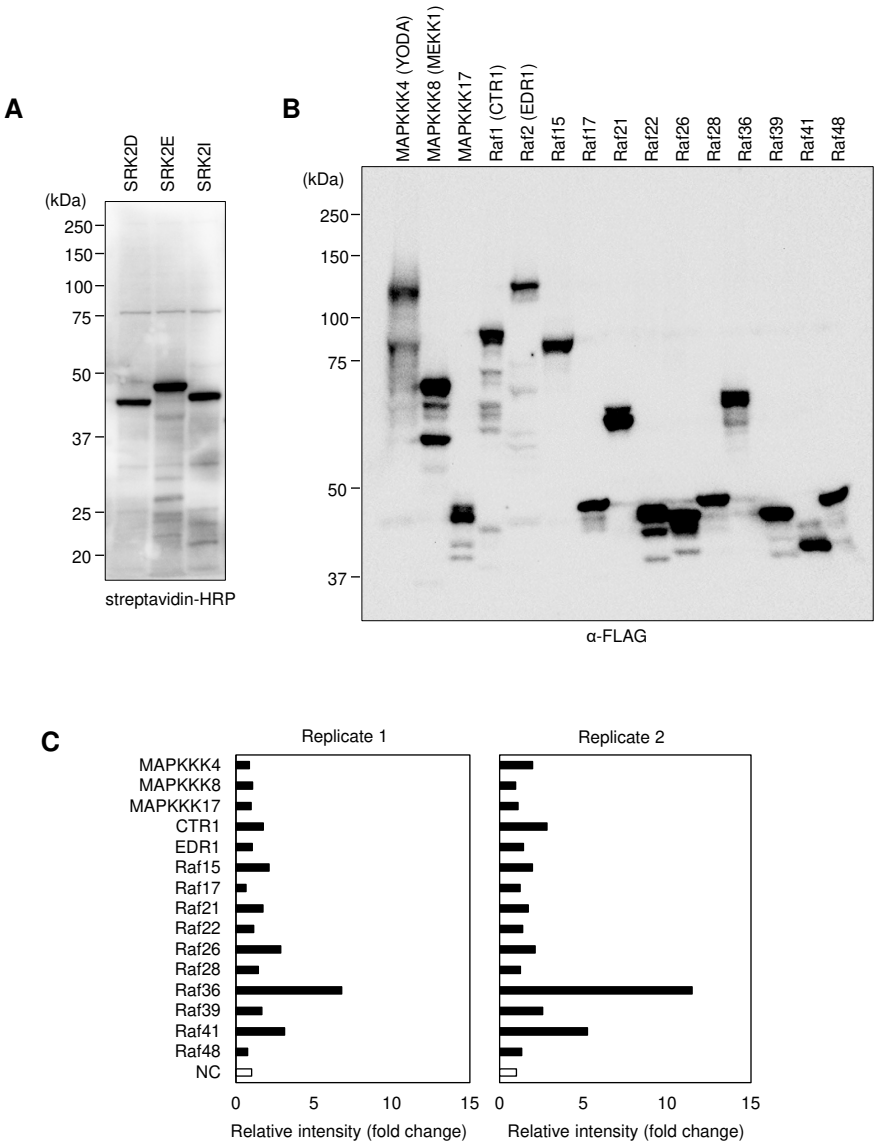

Fig. S1. AlphaScreen® assay for screening of SnRK2-interacting MAPKKKs.
(A and B) N-terminal biotin-tagged SnRK2 proteins and C-terminal FLAG-tagged MAPKKK proteins were synthesized by *in vitro* translation system in wheat germ extracts (WGE). Then extracts were separated by SDS-PAGE (10% gel for SnRK2s, 8% gel for MAPKKKs) and the protein expression was confirmed by western blotting with streptavidin-HRP for SnRK2s (A) or anti-FLAG antibody for MAPKKKs (B). (C) Interaction of SRK2I with MAPKKKs was tested by AlphaScreen® assay. After incubating in a reaction buffer including AlphaScreen® acceptor and donor beads for 12 h, the AlphaScreen® luminescence intensity was analyzed with a multi-mode plate reader (TECAN M1000pro).

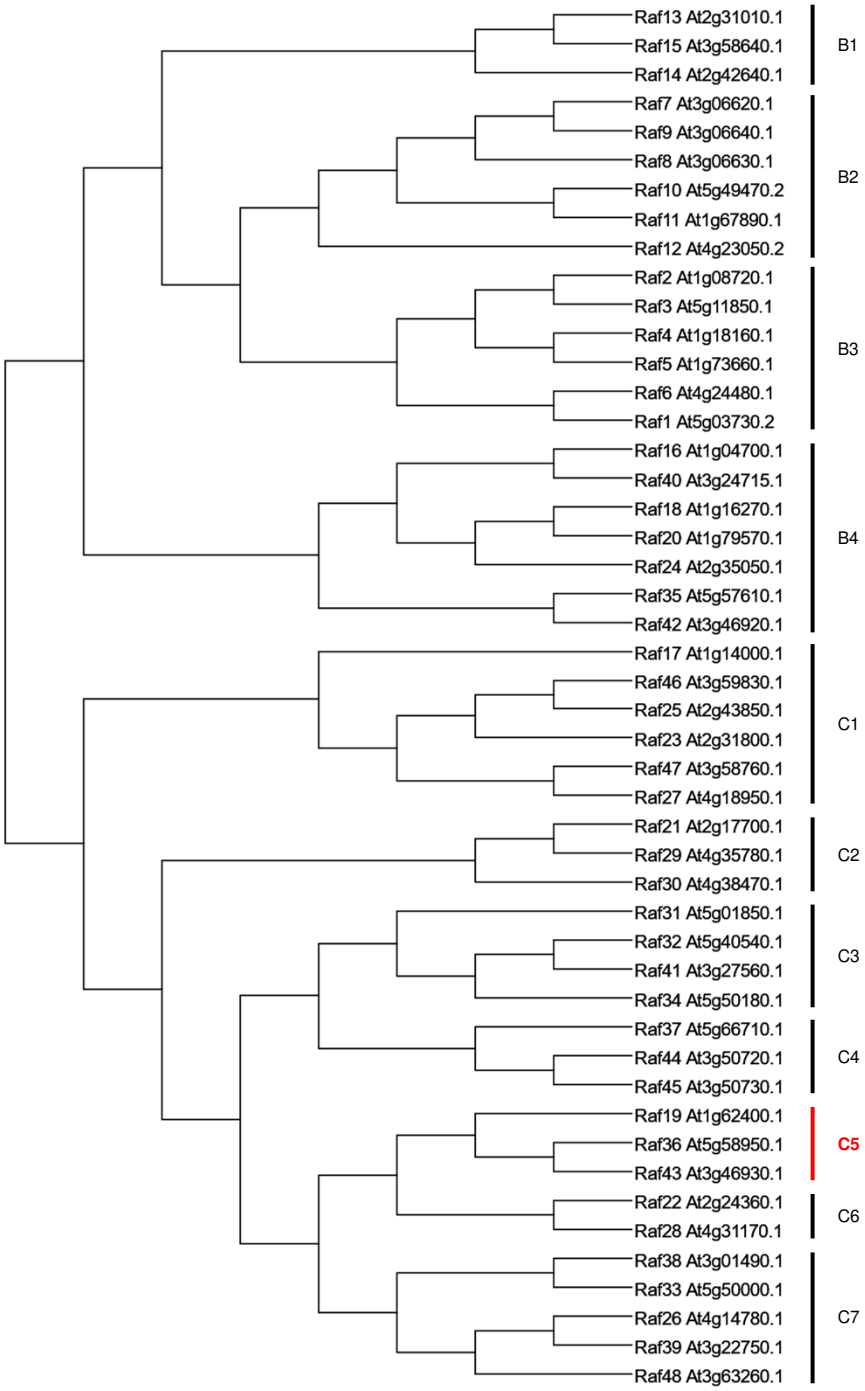

Fig. S2. A phylogenetic tree of Arabidopsis Raf-like kinases.
Amino acid sequences of predicted kinase domains from Raf-like kinases were aligned using ClustalW. The phylogenetic tree was generated using MEGA-X software with the neighbor-joining method. The Raf-like kinases were classified as B1- B4 and C1- C7 subfamilies, according to Ichimura et al., 2002. The C5 subfamily is shown in bold red letters.

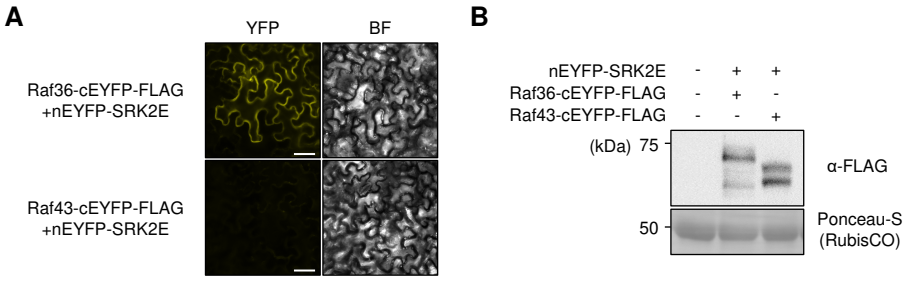

Fig. S3. Raf36, but not Raf43, physically interacts with SRK2E in *N. benthamiana* epidermal cells.
(A) BiFC assays for Raf36 and SRK2E. nEYFP-SRK2E and Raf36-cEYFP-FLAG or Raf43-cEYFP-FLAG were transiently expressed in *N. benthamiana* leaves by Agrobacterium infiltration. nEYFP and cEYFP represent the N- and C-terminal fragments of the EYFP, respectively. BF indicates bright field images. Pictures were taken 3 days after infiltration. Scale bar, 50 μm.
(B) Immunoblot showing the sufficient expression of Raf36-cEYFP-FLAG or Raf43-cEYFP-FLAG proteins in the BiFC experiment. Crude proteins were extracted from *N. benthamiana* leaves at 4 days after Agrobacterium infiltration and then separated by SDS-PAGE. Each Raf protein was detected by western blotting using anti-FLAG antibody. Ponceau-S staining was used as loading control.

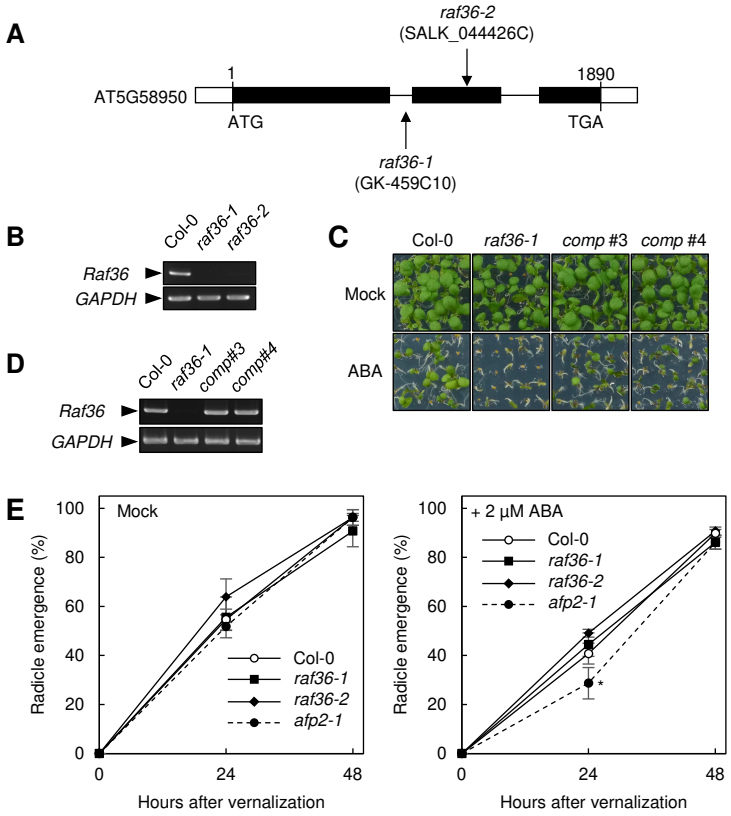

Fig. S4. Isolation and characterization of *raf36-1* and *raf36-2* T-DNA insertion lines.
(A) Schematic depiction of *Raf36* genomic DNA with T-DNA insertions. Black boxes and lines indicate exons and introns, respectively. (B) RT-PCR analysis of *Raf36* transcript levels in wild-type (Col-0), *raf36-1* and *raf36-2* seedlings. *GAPDH* was used as a positive control. (C) Functional complementation of *raf36-1* by *CaMV35S:Raf36-GFP*. Shown is photograph of seedlings grown for 7 days on GM agar medium in the presence or absence of 0.5 µM ABA. (D) RT-PCR analysis of *Raf36* transcript levels in *raf36-1* and complementation lines (*comp*#3 and *comp*#4). *GAPDH* was used as a positive control. (E) Germination rates of wild-type (Col-0), *raf36-1* and *raf36-2* on GM agar medium in the presence or absence of 2 µM ABA were measured by scoring seeds with radicle emergence. Data are means ± standard deviation (n=3), and asterisks showed significant differences (Dunnett's test; **P* < 0.05). Each replicate contains 36 seeds. T-DNA insertion mutant for *ABI FIVE BINDING PROTEIN 2* (*afp2-1; SALK_131676*) was employed as a positive control.

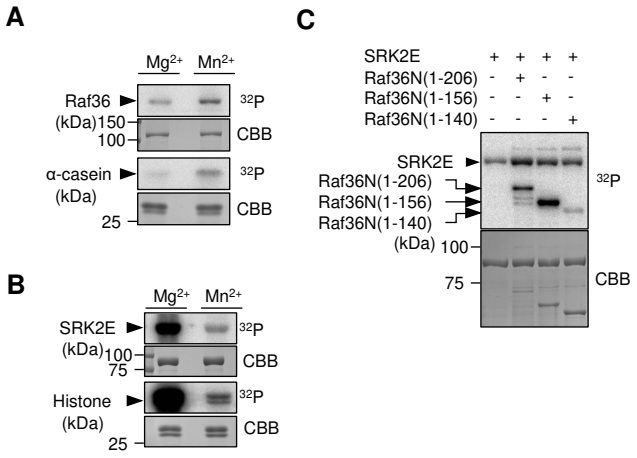

Fig. S5. *in vitro* phosphorylation assays determining preference for Mg^{2+} or Mn^{2+}.
(A) Effects of Mg^{2+} or Mn^{2+} on kinase activity of Raf36. MBP-tagged Raf36 and α-casein were incubated with [γ-^{32}P] ATP in the presence of 5 mM Mg^{2+} (left) or 5 mM Mn^{2+} (right). Autophosphorylation (upper) and substrate phosphorylation (lower) signals were visualized by autoradiography. Coomassie Brilliant Blue (CBB) staining shows protein loading. (B) Effects of Mg^{2+} or Mn^{2+} on kinase activity of SRK2E. MBP-tagged SRK2E and histone as substrate were co-incubated with [γ-^{32}P] ATP in the presence of 5 mM Mg^{2+} (left) or 5 mM Mn^{2+} (right). Autophosphorylation (upper) and substrate phosphorylation (lower) signals were visualized by autoradiography. Coomassie Brilliant Blue (CBB) staining shows protein loading. (C) *In vitro* phosphorylation assay to identify SnRK2-phosphorylation site(s) in the N-terminal region of Raf36.

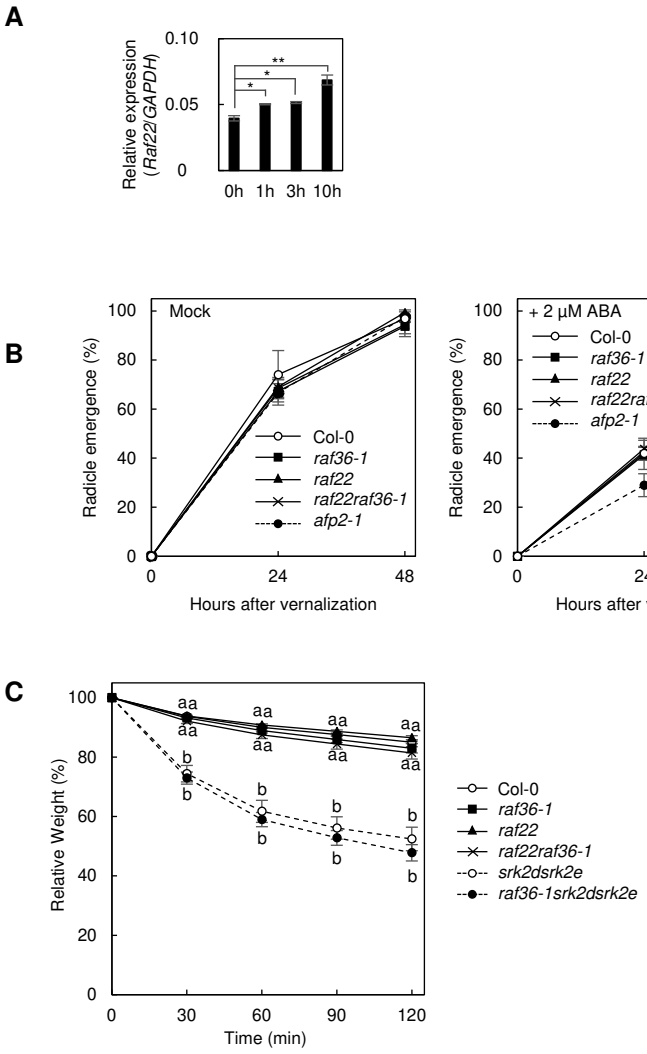

Fig. S6. The characterization of Raf22 in ABA response.
(A) qRT-PCR analysis of *Raf22* mRNA transcript abundance. Total RNA was extracted from 1-week-old wild-type seedlings treated with 50 µM ABA for indicated periods. Bars indicate means ± standard error (n=3) and asterisks showed significant differences by Dunnett's test between ΔCt (non-treated) and ΔCt (ABA-treated) (**P* < 0.05, ***P* < 0.01). (B) Germination rates of wild-type (Col-0), *raf36-1*, *raf22* and *raf22raf36-1* on GM agar medium in the presence or absence of 2 µM ABA were measured by scoring seeds with radicle emergence. Data are means ± standard deviation (n=3), and asterisks showed significant differences (Dunnett's test; **P* < 0.05). Each replicate contains 54 seeds. T-DNA insertion mutant for *ABI FIVE BINDING PROTEIN 2* (*afp2-1*; SALK_131676) was employed as a positive control. (C) Water loss from detached leaves of wild-type (Col-0), *raf36-1*, *raf22, raf22raf36-1* and *raf36-1srk2dsrk2e* plants. The *srk2dsrk2e* mutant was included as a positive control. Data are means ± standard error (n=3) and each replicate consists of five individual leaves. Different letters indicate significant differences for each time point (Tukey's test, *P* < 0.01).

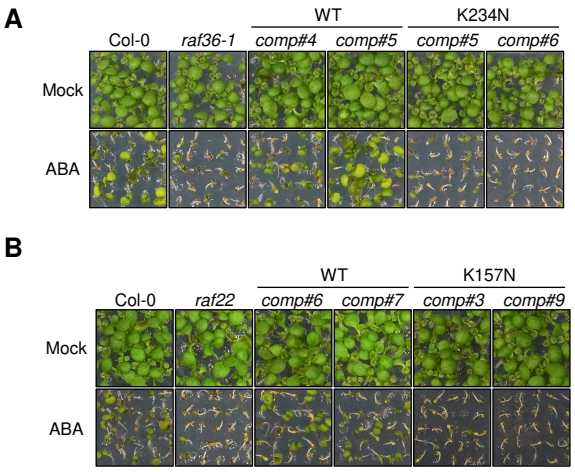

Fig. S7. Raf36 and Raf22 protein kinase activities are required for its function in ABA signaling.
(A) Functional complementation of *raf36-1* by *CaMV35S:Raf36-GFP* or *Raf36 K234N-GFP*. Shown is photograph of seedlings grown for 7 days on GM agar medium in the presence or absence of 0.5 µM ABA. (B) Functional complementation of *raf22* mutant by introducing *CaMV35S:Raf22-GFP* or *Raf22 K157N-GFP*. Shown is photograph of seedlings grown for 7 days on GM agar medium in the presence or absence of 0.5 µM ABA.

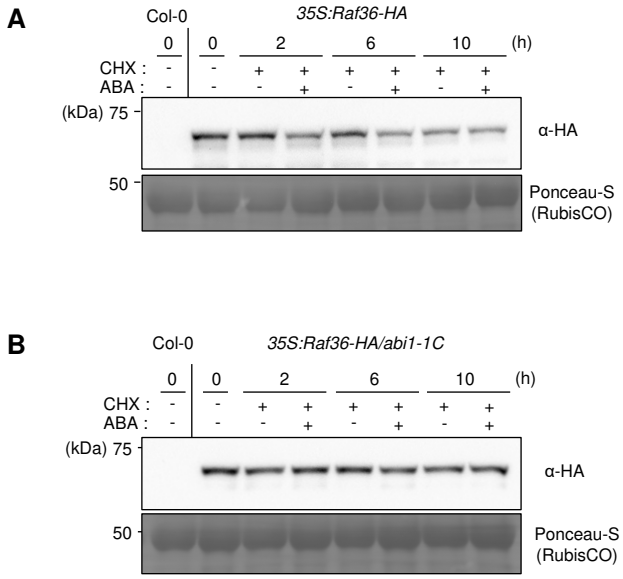

Fig. S8. Western-blot analysis of Raf36-HA after ABA treatment.
(A) Protein extracts were prepared from 2-week-old wild-type (Col-0) or *35S:Raf36-HA* transgenic plants which were treated with only 50 μM CHX or both 50 μM CHX and 50 μM ABA for 0, 2, 6 and 10 h. The Raf36 protein was detected by western blotting using anti-HA antibody. Ponceau-S staining was used as loading control. Similar results were confirmed in the three independent biological replicates. (B) Protein extracts were prepared from 2-week-old wild-type (Col-0) or *35S:Raf36-HA/abi1-1C* plants which were treated with only 50 μM CHX or both 50 μM CHX and 50 μM ABA for 0, 2, 6 and 10 h. The Raf36 protein was detected by western blotting using anti-HA antibody. Ponceau-S staining was used as loading control. Similar results were confirmed in the three independent biological replicates.

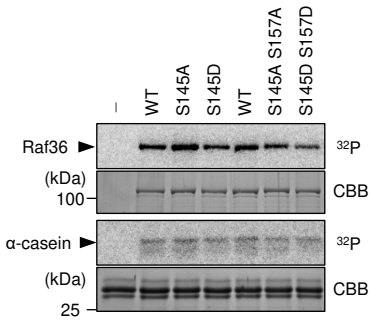

Fig. S9. *in vitro* phosphorylation assay of Raf36 variants.
Each MBP-tagged recombinant Raf36 phospho-variant was co-incubated with α-casein in the presence of [γ-^{32}P] ATP for 30 min. Autophosphorylation (upper) and substrate phosphorylation (lower) signals were visualized by autoradiography (^{32}P). Coomassie Brilliant Blue (CBB) staining shows protein loading.

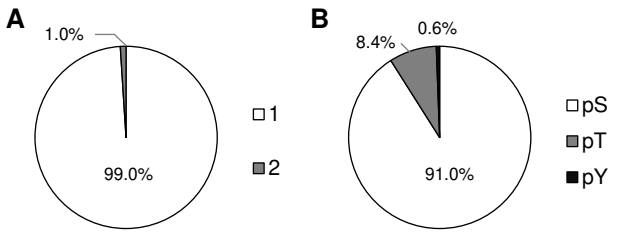

Fig. S10. An overview of phosphoproteomic analysis.
(A) Distribution of the number of phosphosites per peptide.
(B) Distribution of phosphorylated residues in each peptide. *p*S, *p*T and *p*Y indicate phospho-serine, phospho-threonine and phospho-tyrosine, respectively.

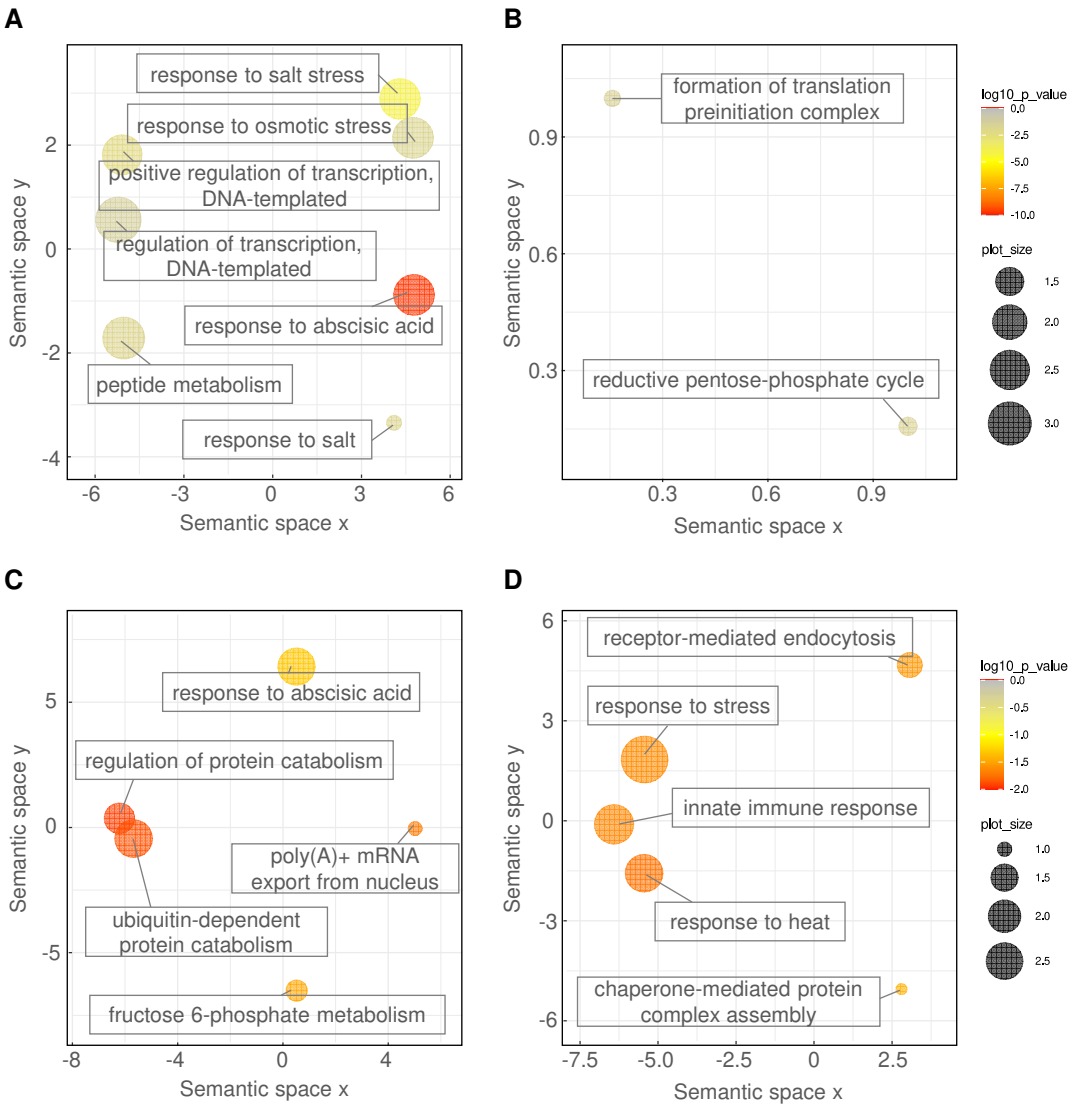

Fig. S11. GO analysis of phosphopeptides in wild-type and *raf22raf36-1*.
Each graph represents GO terms for upregulated (A) and downregulated phosphopeptides (B) in ABA-treated wild-type (WT) seedlings, or GO terms of up- (C) or down-regulated phosphopeptides (D) in *raf22raf36-1* as compared with WT. GO terms were evaluated by DAVID program and visualized with REVIGO ($P < 0.05$). Circle color and size show P value and frequency (%), respectively.

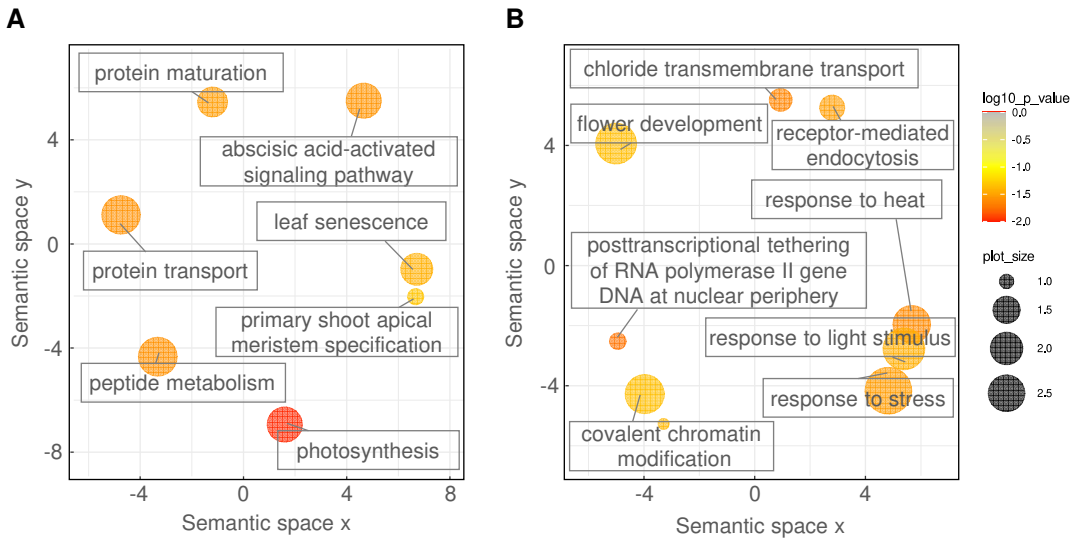

Fig. S12. GO analysis of phosphopeptides in *raf22raf36-1* in normal condition.
Each graph represents GO terms for phosphopeptides up- (A) or down-regulated (B) in *raf22raf36-1* under normal condition ($P < 0.05$). GO terms were evaluated by DAVID program and visualized with REVIGO.

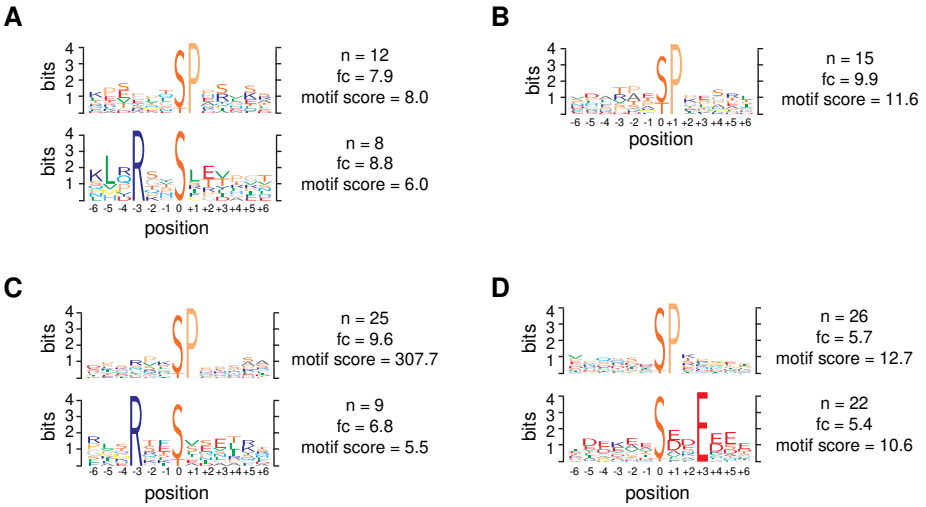

Fig. S13. Motif analysis of phosphopeptides in wild-type and *raf22raf36-1*.
Phosphorylation motifs in up- (A) or down-regulated phosphopeptides (B) in response to ABA in wild-type seedlings, and motifs in up- (C) or down-regulated phosphopeptides (D) in *raf22raf36-1* in comparison with wild-type.

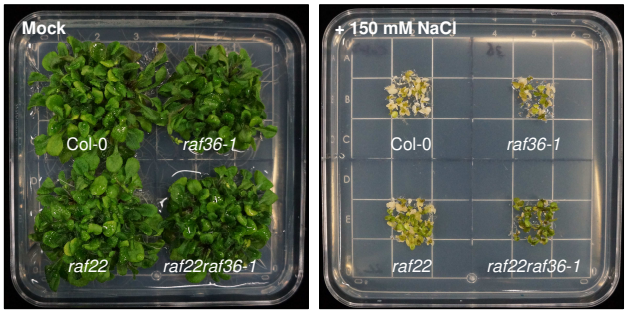

Fig. S14. **Salt tolerance of *raf22raf36-1* plants.**
Wild-type (Col-0), *raf36-1*, *raf22* and *raf22raf36-1* seeds were germinated on GM agar medium with or without 150 mM NaCl. Photographs were taken 19 days after vernalization.

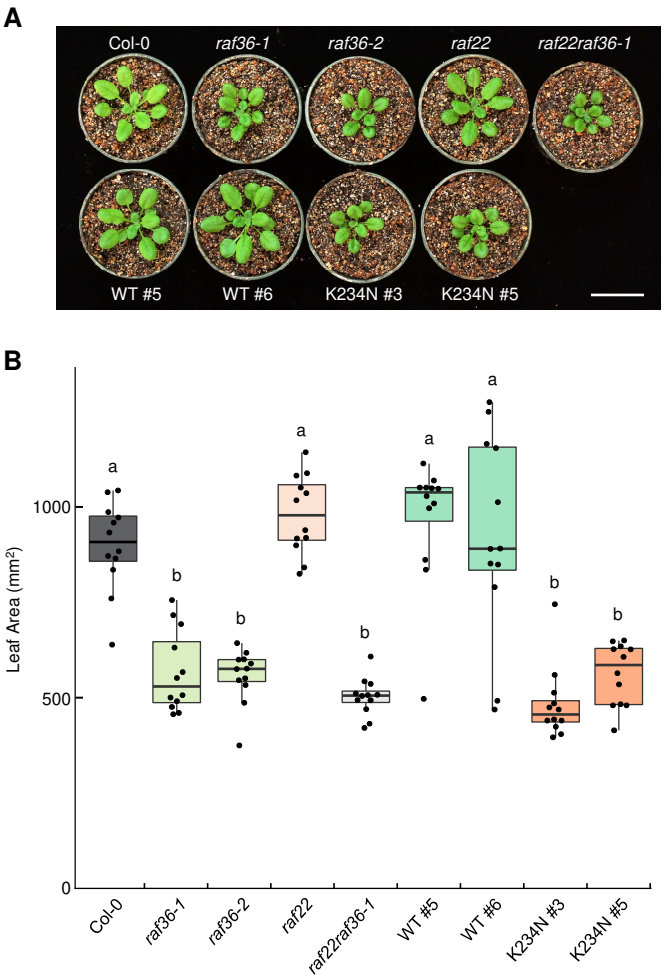

Fig. S15. Dwarf phenotype of *raf36, raf22raf36-1* and *Raf36 K234N* plants under normal condition.
(A) Growth phenotypes of wild-type (Col-0), *raf22, raf36*, *raf22raf36-1*, *Raf36pro:Raf36-3xFLAG/raf36-1* (WT #5 and #6) and *Raf36pro:Raf36 K234N-3xFLAG/raf36-1* (K234N #3 and #5) plants grown at 22° C under 16/8 h photoperiod for 32 days. Scale bar, 3 cm. (B) Comparison of leaf area (mm^2) (box plot; n = 12). Different letters indicate significant differences (Tukey's test, $P < 0.01$).

Table S1. List of predicted molecular weight of recombinant proteins used in this study.
The predicted molecular weight was calculated by summing the theoretical masses of the amino acids.

	Predicted Molecular Weight (kDa)			
	Fused tag protein	Linker	Target protein	TOTAL
MBP-Raf36	40.37	3.23	58.86	102.46
MBP-Raf36 K234N	40.37	3.23	58.84	102.44
MBP-Raf36 S145A	40.37	3.23	58.84	102.44
MBP-Raf36 S145D	40.37	3.23	58.88	102.48
MBP-Raf36 S145A S157A	40.37	3.23	58.82	102.42
MBP-Raf36 S145D S157D	40.37	3.23	58.91	102.51
MBP-Raf36 N (1-206 aa)	40.37	3.23	23.06	66.66
MBP-Raf36 N (1-156 aa)	40.37	3.23	17.40	61.00
MBP-Raf36 N (1-140 aa)	40.37	3.23	15.49	59.09
MBP-Raf36 KD+C	40.37	3.23	35.81	79.41
MBP-Raf36 KD	40.37	3.23	29.92	73.52
MBP-Raf43 K228N	40.37	3.23	57.81	101.41
MBP-Raf22 K157N	40.37	3.23	45.99	89.59
MBP-Raf22 S81A K157N	40.37	3.23	45.97	89.57
MBP-Raf28 K158N	40.37	3.23	46.07	89.67
MBP-SRK2D	40.37	3.23	42.01	85.61
MBP-SRK2I	40.37	3.23	41.08	84.68
MBP-SRK2E	40.37	3.23	41.05	84.65
GST-SRK2E	25.50	1.85	41.05	68.40
GST-SRK2E K50N	25.50	1.85	41.03	68.38
GST-Raf36 (134-163) #1	25.50	1.85	3.43	30.78
GST-Raf36 (134-163) #2	25.50	1.85	3.38	30.73
GST-Raf36 (134-163) #3	25.50	1.85	3.38	30.73
GST-Raf36 (134-163) #4	25.50	1.85	3.38	30.73
GST-Raf36 (134-163) #5	25.50	1.85	3.38	30.73
GST-Raf36 (134-163) #6	25.50	1.85	3.38	30.73
GST-fragment #1 (AT4G25140.1, 143-173 aa)	25.50	1.85	3.61	30.96
GST-fragment #2 (AT3G62800.1, 60-90 aa)	25.50	1.85	3.28	30.63
GST-fragment #3 (AT2G02070.1, 56-86 aa)	25.50	1.85	3.42	30.77
GST-fragment #4 (AT5G04740.1, 124-154 aa)	25.50	1.85	3.34	30.69
GST-fragment #5 (AT5G16260.1, 141-171 aa)	25.50	1.85	3.36	30.71
GST-fragment #6 (AT3G23920.1, 42-72 aa)	25.50	1.85	3.24	30.59
GST-fragment #7 (AT1G70770.1, 72-102 aa)	25.50	1.85	3.56	30.91
GST-fragment #8 (AT3G54610.1, 69-99 aa)	25.50	1.85	3.27	30.62
GST-fragment #9 (AT2G24050.1, 18-48 aa)	25.50	1.85	3.29	30.64
GST-fragment #10 (AT1G37130.1, 48-78 aa)	25.50	1.85	3.60	30.95